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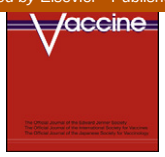

Live oral typhoid vaccine *Salmonella* Typhi Ty21a – A surrogate vaccine against non-typhoid salmonella?

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ABSTRACT

Background: Non-typhoid Salmonella (NTS) is a leading cause of food-borne illness with more than 90 million annual cases and an emerging antimicrobial resistance among the strains worldwide. Paradoxically, no vaccines are available against these pathogens. Numerous NTS strains share surface O-antigens with *Salmonella enterica* serotype Typhi. As intestinal antibodies against O-antigens have proven protective against NTS in animal experiments, it appears conceivable that the oral whole-cell typhoid vaccine, *Salmonella* Typhi Ty21a (Vivotif®), which effectively elicits intestinal antibodies against O-antigens, could exhibit cross-protective efficacy against NTS. We sought immunological evidence in support of cross-protective efficacy of Ty21a against NTS.
Materials and methods: 35 volunteers receiving Ty21a vaccine and five patients with enteric fever were investigated with ELISPOT for circulating plasmablasts secreting antibodies reactive with *Salmonella* Typhi and six different NTS serotypes. These plasmablasts were also analysed for homing receptor expressions.
Results: In all vaccinees and patients, a strong gut-directed cross-reactive plasmablast response was found against serotypes sharing the two O-antigens with *Salmonella* Typhi (O-9,12) (in vaccinees, mean: 95%CI 268: 228–508 and 363: 234–493 plasmablasts/10^6PBMC against *Salmonella* Typhi and Enteritidis). Responses against strains sharing one O-antigen (O-12) were weaker (222: 105–338 against *Salmonella* Typhimurium), while no significant reactivity was detected against strains without typhoidal O-antigens.
Conclusions: Intestinal antibodies against O-antigens protect against NTS in animal experiments. Ty21a was found to elicit intestinal immune responses cross-reactive with NTS strains sharing O-antigens with Ty21a. These include the most common NTS, *Salmonella* Enteritidis and Typhimurium. The data suggest that Ty21a may have cross-protective efficacy against numerous NTS strains.

1. Introduction

Non-typhoid Salmonella (NTS) is a leading cause of food-borne illness both in developing and developed countries, with an estimate of more than 90 million cases of gastroenteritis and 155,000 deaths occurring globally each year [1]. Clinical manifestations of non-typhoidal salmonellosis include gastroenteritis and bacteremia as well as endovascular and local infections [2]. By far the most common manifestation is gastroenteritis, invasive NTS (iNTS) occuring in up to 5% of immunocompetent individuals [3]. In Sub-Saharan Africa the incidence of iNTS appears to be much higher than elsewhere, particularly among children and HIV-infected adults [2–6]. Gastroenteritis is usually self-limited and does not require antibiotics, although in iNTS appropriate antimicrobial therapy can be lifesaving. However, the increasing antimicrobial resistance and emergence of multiresistant strains among NTS pose a serious problem for public health [7,8] and veterinary medicine worldwide [2,9]. This focuses attention on vaccines, yet, there are currently no vaccines against NTS available for clinical use.

Abbreviations: ALS, antibodies in lymphocyte supernatants; ASC, antibody-secreting cell; CLA, cutaneous lymphocyte antigen; HR, homing receptor; iNTS, invasive non-typhoid Salmonella; NTS, non-typhoid Salmonella; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline.

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The current nomenclature divides the genus *Salmonella* into two species, *Salmonella enterica* and *Salmonella bongori* [10,11], the former being further divided into six subspecies. The subspecies *enterica* comprises human-restricted typhoidal serotypes (Typhi and Paratyphi) causing enteric fever (typhoid and paratyphoid fever), and NTS serotypes causing mostly gastroenteritis, usually of zoonotic origin [10,11]. There are more than 2400 NTS serotypes, some of which are closely related both phenotypically and genetically [10]. Interestingly, both *Salmonella* Typhi and the most common NTS, *Salmonella* Enteritidis [10–12], share the same O-antigen profile O-9,12, and even the second most common serotype, *Salmonella* Typhimurium [10,11], shares the O-12 antigen with *Salmonella* Typhi. This opens up an interesting possibility, as one of the currently available typhoid vaccines, the oral live whole cell vaccine *Salmonella* Typhi Ty21a, carries both O-9 and 12 antigens, and has been well documented to induce an intestinal IgA response reactive with these structures [13–16]. It is well established in animal experiments that intestinal or mucosal SIgA antibodies against O-antigen confer protection [17–23]. Thus, it appears that this vaccine could have potential for providing cross-protection against numerous NTS strains, including the two most common types.

Intestinal antibody responses can be studied either by examining secretory IgA in the intestinal secretions or by examining gut-derived plasmablasts from the peripheral blood. The latter approach is based on the recirculation of intestinal lymphocytes after antigen encounter: activated antigen-specific plasmablasts migrate to mesenteric lymph nodes from where they return via the lymphatics and blood to the intestinal lamina propria [24,25]. Detected in the peripheral blood while migrating, approximately one week after antigen encounter, these cells can be used for studying intestinal immune responses [14–16,26,27]. Numerous studies have characterized *Salmonella* Typhi-specific plasmablasts in the circulation after oral Ty21a vaccination [14–16]. All of these plasmablasts carry the intestinal homing receptor, $\alpha_4\beta_7$ [16,26], which acts as an indicator for their migration to the intestinal lamina propria [24,25]. In NTS disease the intestinal immune response appears to be of central importance. The present study explores the cross-reactive potential of Ty21a against NTS strains.

2. Materials and methods

2.1. Study design

Volunteers vaccinated with Ty21a and patients with enteric fever were examined for circulating plasmablasts specific for *Salmonella* Typhi and six different NTS strains. These cells were identified by enzyme-linked immunosorbent spot assay (ELISPOT) as antigen-specific antibody-secreting cells (ASC). In a subgroup of vaccinees, the homing potentials of these cells were characterized by combining immunomagnetic cell sorting with the ELISPOT. Levels of specific antibodies were determined by ELISA in the serum and ALS (antibodies in lymphocyte supernatants) samples of the vaccinees.

The study protocol was approved by the ethics committee of the Helsinki University Central Hospital and the Finnish Medicines Agency and registered in the International Standard Randomised Controlled Trial Number Register (ISRCTN68125331). Written informed consent was obtained from all study subjects.

2.2. Volunteers, vaccinations and samples

Thirty-five healthy Finnish-born volunteers (21 female, 14 male; aged 18–62, mean 32 years) with no history of enteric fever or typhoid vaccination, were given the oral vaccine containing $\geq 2 \times 10^9$ live *Salmonella* Typhi Ty21a bacteria (Vivotif® Crucell NV, Leiden, The Netherlands, lot 3001777), administered one capsule on each of days 0, 2, and 4 as recommended by the manufacturer. The vaccine proved well-tolerated: only 2 out of 35 vaccinees reported stomachache and 1/35 felt nauseated.

The typhoid fever patients (three females, one male; aged 22–30 years) included two Finnish-born travellers returning from Central-America and India, a Sri Lankan applying for asylum in Finland, and a Nepalese immigrant with an infection relapse one month after the first episode. The patient with paratyphoid fever caused by *Salmonella* Paratyphi A (sharing O-12 antigen with *S.* Typhi) was a 30-year-old female immigrant from India. All these patients were treated at the Division of Infectious Diseases, Helsinki University Hospital. Typhoid and paratyphoid fever were diagnosed by blood culture.

In our previous studies on mucosal [14–16] and parenteral [16,28] immunization, ASC have been found to appear transiently in the circulation on day 2–3, peaking on day 7 [14,15]. On the basis of these kinetics, blood samples were drawn before and 7 days after vaccination (ELISPOT and ALS) or 7–10 days after the onset of infection symptoms (ELISPOT). Serum samples were collected before and 28 days after vaccination.

2.3. Antigens

Eight bacterial strains altogether were used as antigens in the ELISPOT assay (Table 1). Four *Salmonella* strains shared at least one O-antigenic determinant (9, 12 or both) with *Salmonella* Typhi, and two harbored different O-antigens (O-6,7 or 6,8). *Yersinia enterocolitica* was used as a negative control. Bacteria were formalin-killed, as described earlier [15], and the concentration of the suspension was adjusted to 10^9 bacteria/mL in PBS (phosphate buffered saline).

2.4. Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were separated using Ficoll-paque density gradient centrifugation as described previously [15].

2.5. Separation of the receptor-negative and -positive cell populations

The expressions of HR on *Salmonella* Typhi and Enteritidis -specific ASC were explored in 15 vaccinees. Separation of the cells into HR-positive and -negative populations has been described earlier [16,26–28]. Briefly, aliquots of cell suspensions (3.4×10^6 PBMC were investigated per HR) were incubated with monoclonal antibodies against $\alpha_4\beta_7$ (ACT-1, Millennium Pharmaceuticals, Cambridge, MA), L-selectin (Leu 8, Becton Dickinson, Erenbodegem-Aalst, Belgium), or CLA (HECA-452, a gift from Dr. Sirpa Jalkanen, Finland). Next, the cells were incubated with Dynal® M-450 magnetic beads coated with sheep anti-mouse IgG (Dynabeads, Dynal Biotech, Oslo), followed by magnetic separation. Separated cells were immediately studied with the ELISPOT assay.

2.6. ELISPOT assay

The isolated PBMC and, for HR analyses, the receptor-positive and -negative cell populations were assayed for antigen-specific ASC using ELISPOT, as described earlier [15]. Briefly, 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with a whole cell preparation of formalin-killed bacteria. The cells were incubated in the wells for 2 h (2×10^6 PBMC/mL, 50 μL/well, total 2.4×10^6 PBMC per antigen), and antibodies secreted were detected with alkaline phosphatase-conjugated goat anti-human IgA (Sigma–Aldrich), IgG (Sigma–Aldrich) and IgM

Table 1
Description of bacterial strains, plasmablast responses to each strain and results of statistical comparisons. The bacterial strains[a] used in the ELISPOT assay, the O-antigens of each strain, the number of plasmablasts (ASC/10^6 PBMC) specific to each strain in 35 volunteers vaccinated one week earlier with the oral Ty21a vaccine (means and 95% confidence intervals), and statistical comparison (Bonferroni corrected Wilcoxon signed rank test) between the responses to various strains. Significant differences are indicated with asterisks (*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$), – = not applicable, NS = not significant.

Bacterial strain	Strain	O- anti-gens	Mean (95%CI)	Comparison with plasmablast response to							
				Salmonella Typhi	*Salmonella* Enteritidis	*Salmonella* Typhimurium	*Salmonella* Agona	*Salmonella* Stanley	*Salmonella* Virchow	*Salmonella* Hadar	*Yersinia enterocolitica*
Salmonella Typhi	Vsa61	9, 12	368 (228–508)	–							
Salmonella Enteritidis	RHS634	1, 9, 12	364 (235–493)	NS	–						
Salmonella Typhimurium	8965	1, 4, 5, 12	222 (105–339)	***	***	–					
Salmonella Agona	RHS6160	4, 12	191 (86–295)	***	***	**	–				
Salmonella Stanley	RHS6766	4, 5, 12	205 (92–319)	***	***	**	NS	–			
Salmonella Virchow	RHS6740	6, 7	2 (0.4–3.4)	***	***	***	***	***	–		
Salmonella Hadar	RHS148	6, 8	1 (0.5–2)	***	***	***	***	***	*	–	
Yersinia enterocolitica	RHI4823	–	0 (0.02–0.4)	***	***	***	***	***	NS	*	–

[a] All strains were from the collection of the Gastrointestinal Infections Unit (formerly Enteric Bacteria Laboratory) of the National Institute for Health and Welfare (formerly National Public Health Institute), Helsinki, Finland.

(SouthernBiotech, Birmingham, England). The substrate (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt; Sigma–Aldrich) was added in melted agarose. The spots were enumerated with an AID Elispot reader, each spot interpreted as a print of one ASC. The specificity, linearity, stability, and intermediate precision of the ELISPOT assay were validated. A response was defined as at least 3 ASC/10^6 PBMC and marked as LOD (limit of detection) in the Figures. This limit has been determined over the assay validation process.

2.7. ALS cultures

PBMC were cultured in RPMI 1640 medium supplemented with 3 μg/mL l-glutamine (2 mM), penicillin (100 μg/mL) and streptomycin (100 μg/mL), and 10% fetal calf serum in flat-bottomed 96-well plates at 37 °C in 5% CO_2 (2×10^6 PBMC/200 μL/well). Supernatants were collected after three days, and stored at −70 °C until assayed.

2.8. ELISA

Antibodies (IgA, IgG and IgM) in serum and ALS samples were measured with ELISA: microtiter plates (Polysorp, Nunc) were coated with a preparation of LPS of *Salmonella* Typhi, Enteritidis, or Typhimurium strains (10 μg/mL 30% methanol-PBS, all from Sigma–Aldrich) overnight and blocked with 5% milk-PBS solution. Next, the samples were serially diluted in the blocking solution, and serum samples were added in the wells at a dilution of 1:1500, and ALS samples at 1:150, and incubated overnight. The horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA, IgG and IgM antibodies (Dako) were added (2 h at room temperature), and the color developed with TMB peroxidase substrate (3,3′,5,5′- tetramethylbenzidine and H_2O_2 in citric acid buffer; KPL, Gaithersburg, USA) and stopped with 0.5 M sulfuric acid. A response was defined as at least two-fold increase in the titre from the prevaccination level in the specific antibody titres.

2.9. Statistics

Statistical analyses were carried out with JMP software version 9.0.0 (SAS Institute Inc., Cary, NC, USA). The distributions of the ASC and HR expressions were tested with Shapiro–Wilk's test. Since not all distributions proved normal even after log transformations, Bonferroni-corrected Wilcoxon-signed rank test was used for comparisons between the multiple groups, and Wilcoxon-signed rank test for comparisons between two groups. Correlation analyses were performed using the Spearman test. The results are given as the means, medians, and 95% confidence intervals (CI) for the number of ASC, and as means ±95%CI for the HR expressions. Statistical comparisons are presented (a) to reveal significant responses (comparisons with a negative control, *Yersinia enterocolitica*; Table 1), (b) to reveal differences to a strain against which Ty21a has in field trials been shown to provide protection (*S.* Typhi; Table 1, Fig. 2), and (c) to reveal differences in the magnitude of the responses between *Salmonella* strains sharing both O-9,12 or only O-12, or no O-antigens with the Ty21a strains (various NTS strains; Table 1). The proportions of the receptor-positive ASC were calculated as follows: percentage of receptor-positive ASC = (100 × the number of ASC in receptor-positive cell population)/(the sum of ASC in receptor-positive and -negative cell populations).

3. Results

3.1. The ASC response in volunteers vaccinated withTy21a

Before vaccination, no ASC specific to *Salmonella* Typhimurium, Agona, Stanley, Virchow, or *Yersinia enterocolitica* were found in

the circulation of any of the vaccinees (Fig. 1). One volunteer had 5 ASC/10^6 PBMC reactive with *Salmonella* Typhi and Enteritidis, and another 40 ASC/10^6 PBMC reactive with *Salmonella* Hadar before vaccination (Fig. 1). Seven days after vaccination, a substantial number of circulating *Salmonella* Typhi and Enteritidis-specific ASC were detected in all vaccinees. 32 out of 35 showed a response to *Salmonella* Typhimurium and 31/35 to Agona, and Stanley (Fig. 1, Table 2), 6/35 and 5/35 a minor response (less than 20 ASC/10^6 PBMC) to Virchow and Hadar, respectively, and none to *Yersinia enterocolitica*. The highest cross-reactive responses were seen to *Salmonella* Enteritidis sharing both O-antigens with *Salmonella* Typhi, and substantial responses were found also against strains only sharing one O-antigen, such as *Salmonella* Typhimurium (Figs. 1 and 2, Table 1). The responses were mostly dominated by IgA and IgM (data not shown).

3.2. The ASC response in patients with enteric fever

All patients with enteric fever had ASC specific to *Salmonella* Typhi, Enteritidis, Typhimurium, Agona, and Stanley (Fig. 3).

3.3. The expression of $\alpha_4\beta_7$, L-selectin and CLA on specific ASC

Almost all ASC expressed the intestinal HR, $\alpha_4\beta_7$-integrin, whereas the peripheral lymph node HR, L-selectin, and the cutaneous HR, CLA, were expressed less frequently (Fig. 4). No differences were seen between the HR expressions on plasmablasts specific to the two bacterial strains.

3.4. Antibodies in ALS and serum samples

The numbers of vaccinees responding with a twofold or higher rise in antibody titres in ALS and serum samples was smaller than those responding in the ELISPOT assay (Table 2). With all antigens investigated, there was an excellent correlation between the magnitudes of the IgA responses in the two plasmablast assays, ALS and ELISPOT (*S.* Typhi $r = 0.942$ $p < 0.001$; *S.* Enteritidis: $r = 0.9665$, $p < 0.01$; *S.* Typhimurium: $r = 0.9837$, $p < 0.05$). Less consistency was found between the IgM responses: some correlation was seen between ALS and ELISPOT against *S.* Typhi and *S.* Enteritidis ($r = 0.625$, $p < 0.001$ and $r = 0.3809$, $p < 0.05$, respectively), but not between the weak IgM responses to *S.* Typhimurium. No correlation was detected between the IgG responses in the ALS and ELISPOT assays with any of the three strains. Responses in the plasmablast assays showed no correlation with serum antibody assays for any of the three antigens.

4. Discussion

The present study shows significant cross-reactive immune responses against NTS strains in enteric fever and after Ty21a vaccination in humans. The results suggest that Ty21a vaccine could potentially confer cross-protection against numerous NTS strains.

The immunological background accounting for the cross-reactive immune response elicited by Ty21a against NTS is suggested to be based on shared epitopes among the O-antigens. Ty21a and *Salmonella* Typhi both carry O-9,12. Of the NTS strains, some share both of these O-antigens (e.g. *Salmonella* Enteritidis) with Ty21a, while others share only the O-12 epitope (e.g. *Salmonella* Typhimurium, Agona, and Stanley) or none at all (e.g. *Salmonella* Hadar and Virchow). Consistent with this, the cross-reactive plasmablast response to *Salmonella* Enteritidis sharing two O-antigens was as vigorous as that to *Salmonella* Typhi, whereas the responses to strains sharing only one O-antigen proved somewhat lower. No significant cross-reactivity was seen to strains not sharing any O-antigens. The small number of plasmablasts reactive

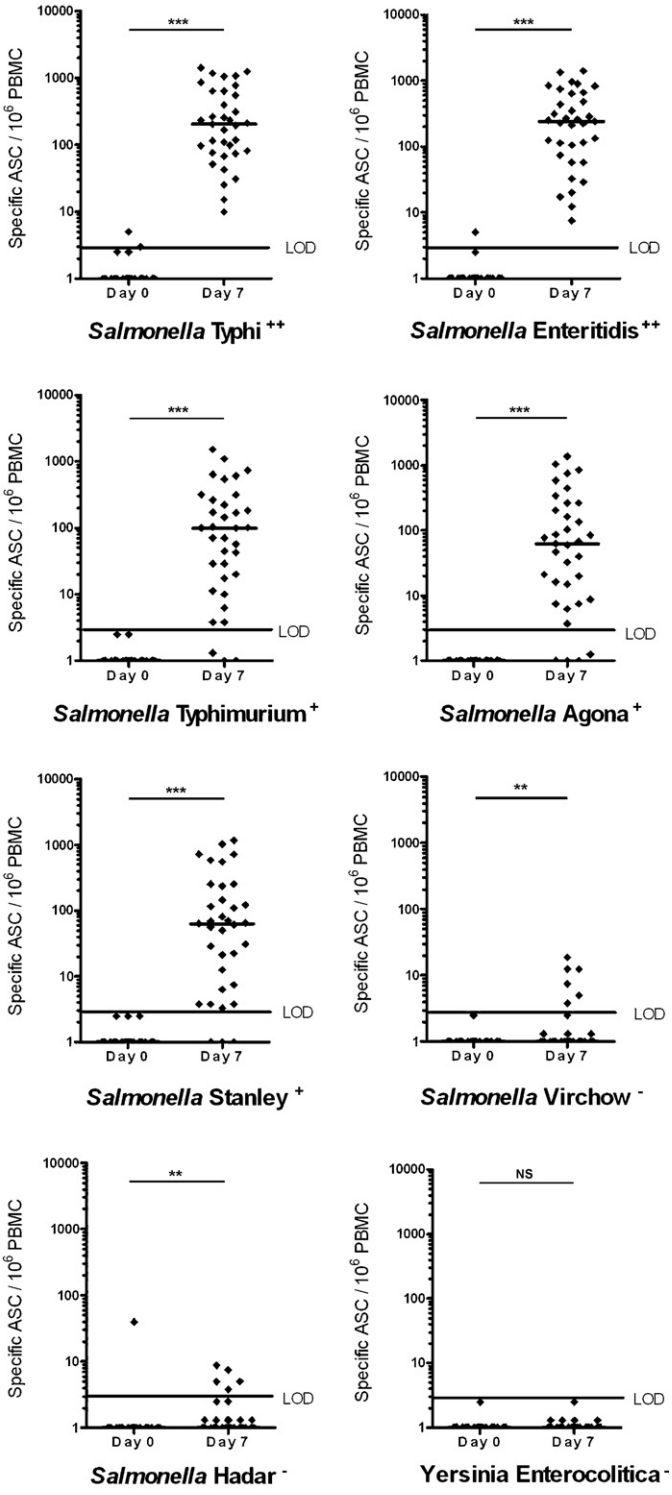

Fig. 1. Plasmablast response to NTS strains on days 0 and 7 after Ty21a vaccination. Numbers of circulating antigen (*Salmonella* Typhi, Enteritidis, Typhimurium, Agona, Stanley, Virchow, or Hadar and *Yersinia enterocolitica*)-specific plasmablasts, identified as antibody-secreting cells (ASC) in 35 volunteers vaccinated with the oral Ty21a vaccine. The dots represent results of individual vaccinees and the lines the medians of the numbers of Ig(A + G + M)-plasmablasts on day 0 and 7 after vaccination. (LOD = lower limit of detection). Statistical comparison (Wilcoxon signed rank test) has been indicated with asterisk (*** $p < 0.001$; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$). The upper indexes +/− indicate strains with (−) no O antigen, (+) one O-antigen (O-12) or (++) two O-antigens (O-9 and O-12) in common with Ty21a.

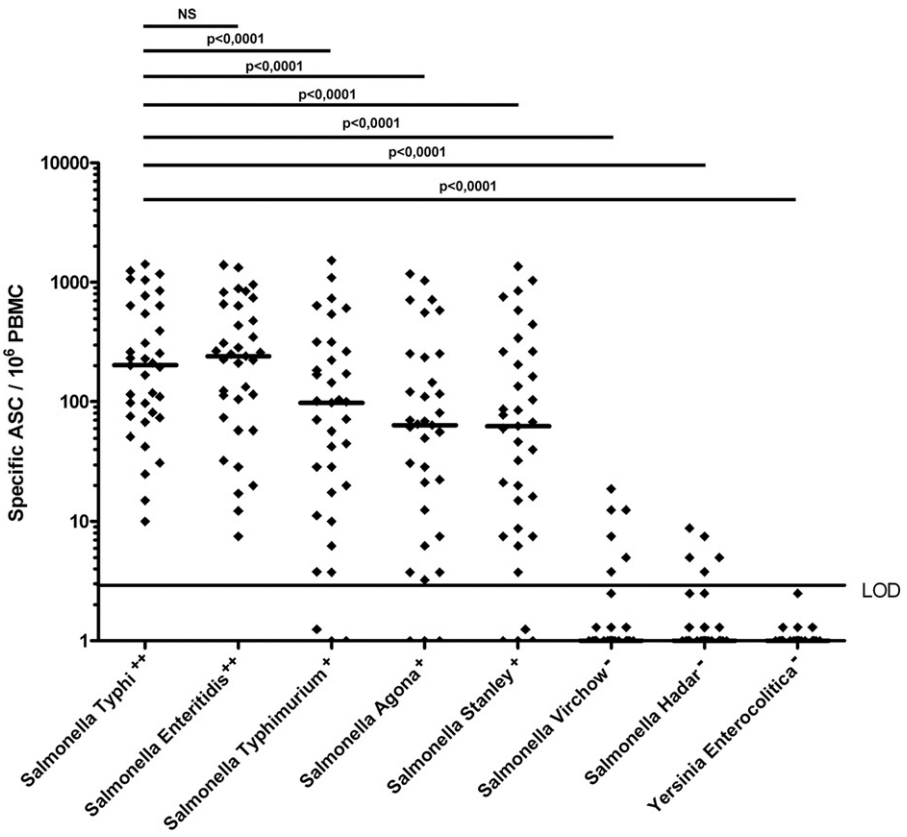

Fig. 2. Comparison of the typhoid-specific and cross-reactive plasmablast responses in volunteers vaccinated with Ty21a. Comparison of the numbers of circulating *Salmonella* Typhi-specific ASC, with the numbers of ASC cross-reacting with *Salmonella* Enteritidis, Typhimurium, Agona, Stanley, Virchow, Hadar, and *Yersinia enterocolitica* in 35 volunteers vaccinated with the oral Ty21a vaccine. The dots represent results of individual vaccinees and the lines the medians of the numbers of Ig(A + G + M)-plasmablasts on day 7 after vaccination. (LOD = lower limit of detection). The *p*-values of statistical comparisons (Bonferroni corrected Wilcoxon signed rank test) between the strains are given above the bars. The upper indexes +/− indicate strains with no O antigen (−), one O antigen (O-12) (+) or two O antigens (O-9 and O-12) (++) in common with Ty21a.

with *Salmonella* Virchow and Hadar in some vaccinees was presumably due to the presence of some minor common antigens, such as some protein structures; using whole bacteria as antigens allows the detection of all potentially cross-reactive antigens.

Notably, the causative agents of paratyphoid fever, *Salmonella* Paratyphi A and B also share the O-12 antigen with *Salmonella* Typhi. Consistently, we have found a cross-reactive plasmablast response [29] and others have described cross-reactive cell-mediated immune responses [30,31], and even protective efficacy of 49% by Ty21a against Paratyphi B in field trials [32]. In fact, we also determined the responses to *S.* Paratyphi A and B in the volunteers participating in the present study (data not shown). Corresponding to the paratyphoid strains only sharing one O-antigen with Ty21a, the magnitudes of the responses to paratyphoid strains were either lower ($p < 0.01$ for *Salmonella* Paratyphi A) than or equal (p – NS for *S.* Paratyphi B) to the same persons' responses against NTS strains sharing only one O-antigen with Ty21a. The responses to paratyphoid strains were significantly lower than those against *Salmonella* Enteritidis sharing two O-antigens with Ty21a ($p < 0.001$ for both *S.* Paratyphi A and B).

The present study is the first to explore cross-reactive plasmablasts against NTS strains in patients with typhoid or paratyphoid fever. Cross-reactive plasmablasts were found in all of these patients, the profile matching with the O-antigens shared between the pathogens. It would be interesting to explore whether enteric fever is followed by a period of protection against NTS strains bearing typhoidal O-antigens.

As the cross-reactive ability of Ty21a does not cover all the NTS strains, the clinical significance of the potential cross-protectivity depends on how common the strains carrying O-9 or O-12 antigens are. No cross-protection can be expected against strains without these typhoidal O-antigens. *Salmonella* Enteritidis, Heidelberg, and Typhimurium have been reported as the most common serovars among invasive *Salmonella* infections in Finland, Australia,

Table 2
Antibody responses in serum and ALS (antibodies in supernatant)-cultures. Numbers of vaccinees responding in serum, ALS culture and ELISPOT assays for *Salmonella* Typhi, Enteritidis and Typhimurium. In the ELISA assays (serum and ALS), a responder was defined as an individual with at least two-fold increase in titre (in IgA, IgG and/or IgM isotype). In the ELISPOT assay, a responder was defined as having at least 3 ASC/10^6 PBMC on day 7. Samples for ELISPOT and ALS were collected on days 0 and 7, and serum samples on days 0 and 28 after Ty21a vaccination. The number of vaccinees tested with each assay is indicated in the Table.

Salmonella enterica serotype	Responders in the assay for											
	Serum antibodies/ELISA 25 vaccinees				ALS/ELISA 13 vaccinees				ASC/ELISPOT 35 vaccinees			
	IgA	IgG	IgM	IgA/IgG/IgM	IgA	IgG	IgM	IgA/IgG/IgM	IgA	IgG	IgM	IgA/IgG/IgM
Typhi	7	5	12	12 (48%)	11	6	10	11 (85%)	35	28	32	35 (100%)
Enteritidis	6	6	11	11(44%)	11	4	7	11 (85%)	33	26	33	35 (100%)
Typhimurium	3	1	5	5 (20%)	7	3	3	7 (54%)	28	22	26	33 (94%)

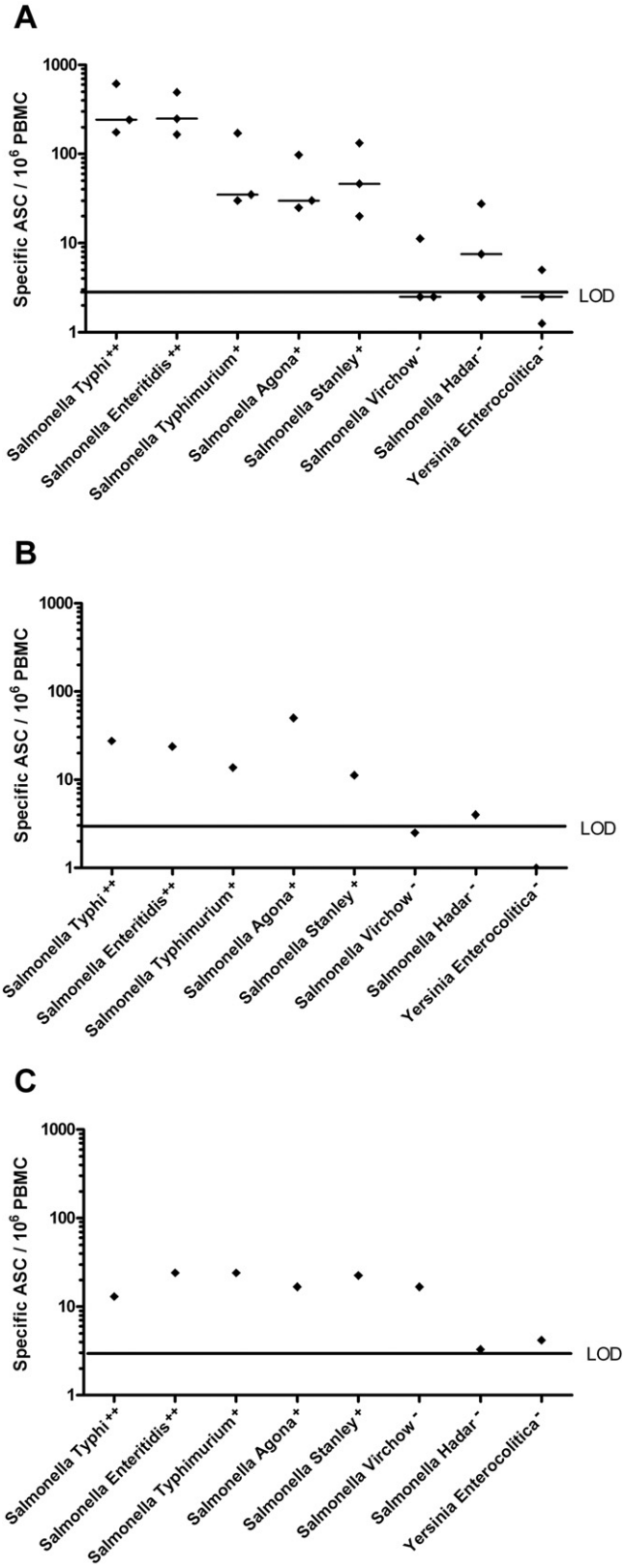

Fig. 3. Pathogen-specific and cross-reactive plasmablast responses in patients with enteric fever. Numbers of circulating antigen (*Salmonella* Typhi, Enteritidis, Typhimurium, Agona, Stanley, Virchow, or Hadar, and *Yersinia enterocolitica*)-specific plasmablasts (ASC) in three patients with typhoid fever in Panel A, one patient with relapsing typhoid fever in Panel B, and one with paratyphoid A fever in Panel C. The dots represent the individual numbers, and the lines the medians of Ig(A + G + M) ASC/10^6 PBMC on day 7–10 after the onset of infection symptoms. (LOD = lower limit of detection). The upper indexes +/− indicate strains with no O antigen (−), one O antigen (O-12) (+) or two O antigens (O-9 and O-12) (++) in common with Ty21a.

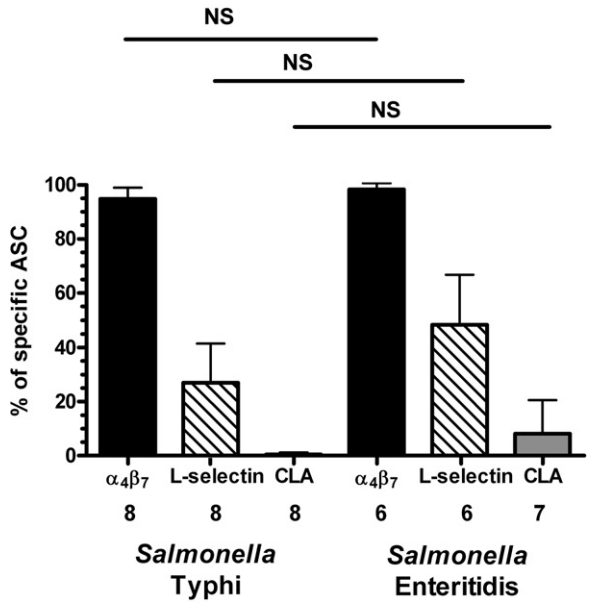

Fig. 4. Homing potentials of *Salmonella* Typhi and Enteritidis-specific plasmablasts after oral vaccination with Ty21a. The expression of $\alpha_4\beta_7$, L-selectin and CLA on *Salmonella* Typhi and Enteritidis -specific plasmablasts in peripheral blood of volunteers seven days after oral vaccination with Ty21a. The bars indicate the arithmetic means + 95%CI of percentages of HR-positive ASC among all pathogen specific ASC (IgA + IgG + IgM). The HRs and the numbers of volunteers from whom the data were pooled are indicated under the data bars. The statistical comparisons were carried out with Wilcoxon signed rank test. NS = not significant.

Denmark and Canada [33]. All these strains express the typhoidal O-antigens. In the EU *Salmonella* has been reported as the main cause of food-borne outbreaks with 165 000 confirmed human cases annually with *Salmonella* Enteritidis and Typhimurium representing 75% of these [11]. In the UK, in 2010, *Salmonella* Enteritis was the most commonly reported serotype in humans (29%), followed by *Salmonella* Typhimurium (23%), yet the numbers of *Salmonella* Enteritidis have been reported to have decreased significantly from the previous year (-36%) [34]. On the other hand, in the USA the incidence of *Salmonella* Enteritidis has been reported to be increasing (44% rise from 1996–1999 to 2006–2009), with domestic cases constituting the great majority [12]. In 2009, out of 40 828 human *Salmonella* cases reported to CDC, *Salmonella* Enteritidis represented the most common strain (17%), and *Salmonella* Typhimurium ranked second (15%) [10]. Antimicrobial resistance is increasing and of the total of 1,8% nalidixic acid resistant strains, the most common serotypes were Enteritidis (38%) and Typhimurium (21%) [10]. On the whole, cross-reactivity with Ty21a is expected to cover about half of all strains reported to CDC: approximately half of them carried typhoidal O-antigens, one quarter both O-antigens 9 and 12, and one quarter only O-12 [10].

As the cross-reactivity mostly appears to be based on a response against O-antigens, data on the protective efficacy of O-antigen specific antibodies are of particular interest. Numerous animal experiments focus on this issue. Intestinal or mucosal secretory IgA (SIgA) against *Salmonella* Typhimurium O-antigens have proved protective [17–23] even in the absence of other mechanisms, such as cell-mediated immune mechanisms [19]. Michetti and colleagues first showed that Sal4, a monoclonal, polymeric IgA antibody against the *Salmonella* O-antigen transported into the intestinal tracts of mice was sufficient to protect the animals against an otherwise lethal oral dose of *Salmonella* Typhimurium [19]. Later Sal4 was found to block the invasion of *Salmonella* Typhimurium into epithelial cell monolayers, and thus prevent the earliest steps of infection [20]. Sal4 was suggested to function by “immune

exclusion" [20]. Forbes et al. [21] recently proposed that Sal4, by cross-linking the O-antigen, compromises the integrity of the bacterial cell envelope rendering the pathogen temporarily avirulent. Importantly, Sal4 transported intraperitoneally has not proved to protect the mice [19], suggesting that these antibodies are protective only at the mucosal site where they can block the first steps of the infection. Consistently, in a recent study, parenterally immunized mice did not mount detectable levels of O-antigen-specific SIgA and none of the mice were protected against wild type *S.* Typhimurium [22]. While studies by Michetti et al. [19] show protection against invasive disease in mice, Endt et al. have also shown in mice immunized with *S.* Typhimurium an adaptive immune response providing protection against a mucosal disease on re-infection with the pathogen in an O-antigen-dependent way [22]. In humans, even if the present study provides the immunological background for a potential protection by Ty21a against various NTS strains in humans, efficacy studies are needed to separately assess the potential protection against local and invasive disease.

The homing profiles of *Salmonella* Enteritidis- and Typhi-specific plasmablasts were similar, as was expected, these cells presumably being the same Ty21a-specific cells, simply showing cross-reactivity with NTS. A pronounced targeting to the intestine was observed, as interpreted by the very high proportion of cells expressing the intestinal HR $\alpha_4\beta_7$ and lower proportion of cells expressing L-selectin. We have previously demonstrated that homing profiles of plasmablasts depend on the site of antigen encounter: typhoid-specific plasmablasts exhibit an intestinal homing profile after oral administration of Ty21a, while a parenterally administered whole cell Ty21a vaccine induces plasmablasts with a systemic homing profile [16]. Consistently, we have also shown an intestinal homing profile in diarrhea caused by NTS strains [26,27]. An intestinal homing profile appears particularly beneficial with respect to the intestinal transmission route of NTS diseases, the findings in animal experiments suggesting that only intestinal antibodies against O-antigens prevented infections with *Salmonella* Typhimurium [19,22].

While serum antibodies provide information on the systemic immune response, the plasmablast assays (ELISPOT and ALS) illustrate an earlier stage of the response representing thus both systemic and mucosal parts; in this case the latter mostly implies an intestinal response as seen from the homing profile. Consistently, the magnitudes of the responses in ALS and ELISPOT assays correlated with one another, but not with the serum antibody responses. Notably, all of these assays supported cross-reactivity. Consistent with previous studies [15], the ELISPOT assay, representing assessment at a single-cell level, proved the most sensitive approach in the evaluation of cross-reactivity.

5. Conclusions

As NTS remains a health problem of global proportions and antimicrobial resistance is increasing, vaccines for clinical use are desperately needed. The present study is the first to show that the Ty21a vaccine currently available elicits a humoral cross-reactive gut-directed, i.e. an intestinal immune response to numerous NTS strains sharing O-antigens with *Salmonella* Typhi. Similar cross-reactive responses were found in patients with enteric fever. The NTS strains include the two most commonly encountered strains *Salmonella* Enteritidis and Typhimurium as well as many others. In animal experiments, intestinal antibodies against O-antigens have proved protective. Taken together, these data provide immunological evidence for cross-protective capacity of the Ty21a vaccine against numerous NTS strains, encouraging future efficacy studies. Any degree of cross-protective capacity in a currently available vaccine would be of tremendous value.

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Conflicts of interest: AK has participated as a member in advisory boards of Pfizer, GlaxoSmithKline and Novartis and received honorarium from these. She has acted as a consultant to Crucell on vaccination immunology and been reimbursed for giving lectures by Crucell, GSK and Bayer. SHP, RK, AS, and JMK declare no conflicts of interest.

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